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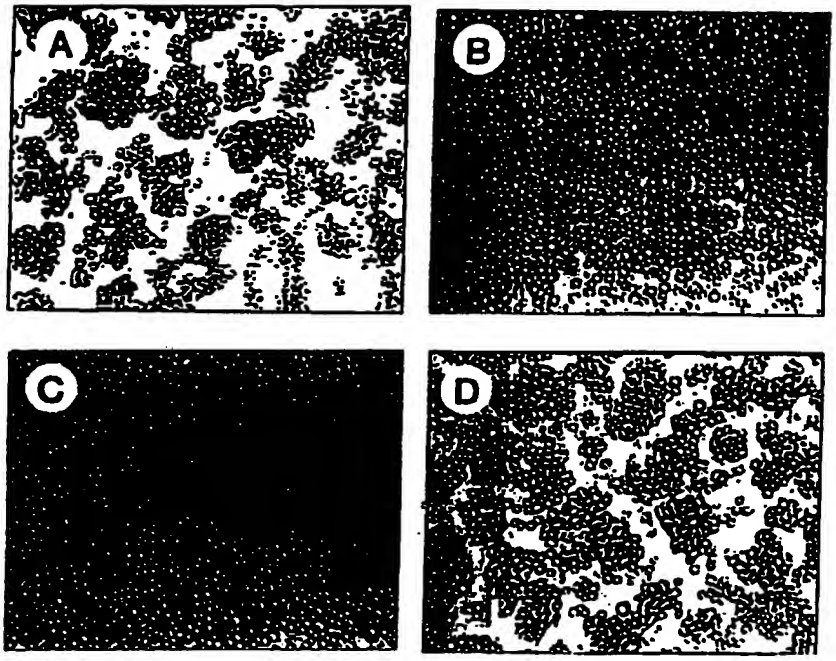
(54) Title: INHIBITION OF CELL ADHESION USING INTERCELLULAR ADHESION MOLECULE-1-LIKE PEPTIDES AND/OR ANALOGUES THEREOF



Abstract

The present invention is directed to Intercellular Adhesion Molecule-1-like peptides, analogues thereof and antibodies eto, to pharmaceutical compositions comprising same and the use thereof to inhibit or reduce cell adhesion in mammals. The sent invention also relates to a method for fingerprinting the functional domain of a polypeptide or protein.

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INHIBITION OF CELL ADHESION USING INTERCELLULAR ADHESION MOLECULE-1-LIKE PEPTIDES AND/OR ANALOGUES THEREOF.

The present invention is directed to Intercellular Adhesion Molecule-1-like peptides, analogues thereof and antibodies thereto, to pharmaceutical compositions comprising same and the use thereof to inhibit or reduce cell adhesion in mammals. The present invention also relates to a method for fingerprinting the functional domain of a polypeptide or protein.

The specific interaction between the two cell surface glycoproteins, Intercellular Adhesion Molecule-1 (hereinafter referred to as "ICAM-1") and Leukocyte Function Associated antigen-1 (hereinafter referred to as "LFA-1") has been shown to be a major adhesive mechanism for haemopoietic and lymphoid cells (Wawryk et al, 1989). Studies using both ICAM-1 and LFA-1 antibodies have shown that the LFA-1/ICAM-1 interaction is critically involved in a wide variety of adhesion-dependent leukocyte functions (Wawryk et al, 1989). Cytokines involved in the "inflammatory" response, IFN-γ, TNFα and IL-1, induce expression of ICAM-1 (Pober et al, 1987: Dustin et al, 1988; Boyd et al, 1989a,b; Campbell et al, 1989).

ICAM-1 has been included as a member of the immunoglobulin supergene family (Staunton et al, 1988; Simmons et al, 1988; Williams and Barclay, 1988). The receptor for ICAM-1, LFA-1, belongs to the structurally different integrin family of proteins. The integrins are heterodimeric proteins, a number of which, bind via the tripeptide RGD motif in their ligands (Ruoslahti and Pierschbacher, 1987). There is no RGD sequence in ICAM-1

(Staunton et al, 1988; Simmons et al 1988), nor does the GRGDSP peptide inhibit ICAM-1/LFA-1 binding (Marlin and Springer, 1987).

Several studies using monoclonal antibodies directed against both ICAM-1 and LFA-1 have shown the ICAM-1/LFA-1 linkage to be of critical importance in the homotypic adhesion of haemopoietic cells (Makgoba et al, 1988; Boyd et al, 1989a), migration of lymphocytes (Dustin and Springer, 1988; Boyd et al, 1989c) and in cell-cell contact-mediated immune mechanisms such as T and B cell activation (Boyd et al, 1988; Dougherty et al, 1988; Altmann et al 1989) and in certain effector functions of the immune response including T-cell cytotoxicity and non-specific cytotoxic cell activity (Boyd et al, 1988; Dougherty et al, 1988; Mentzer et al 1989). This central role of ICAM-1 in cell-cell contact mediated-immune mechanisms and the identification of ICAM-1 as the major human rhinovirus receptor (Greve et al, 1989; Staunton et al, 1989a) and as a receptor for red blood cells infected with Plasmodium falciparum (Berendt et al, 1989), has raised the possibility that ICAM-1 might be a target for therapy. Blocking of ICAM-1 function may be beneficial in the treatment of diseases including autoimmune disorders, graft versus host disease, graft rejection, severe allergic reactions, the common cold and malaria.

Additionally, in work leading up to the present invention, possible binding domains within the ICAM-1 sequence were sought by comparing a hydrophobicity analysis (Kyte and Doolittle, 1982) of ICAM-1 with a dot matrix sequence comparison (Maizel and Lenk, 1981) of ICAM-1 with a structurally similar but functionally distinct protein, Myelin-Associated Glycoprotein (MAG). In accordance with the present invention, a unique region of minimal identity within the ICAM-1 sequence was determined. Furthermore, as disclosed herein, a

synthetic peptide based on this sequence mimics the effects of anti-ICAM-1 antibody in inhibiting ICAM-1/LFA-1-mediated adhesion.

The present invention provides ICAM-1-like peptides and analogues thereof having anti-adhesive properties. More particularly, the ICAM-1-like peptides and their analogues are capable of inhibiting or reducing cell adhesion in mammals. Preferably, the mammal is a human, ruminant animal or domestic animal. Most preferably, the mammal is a human.

By "ICAM-like peptides" in accordance with this invention and as used in the present specification and claims, is meant a peptide, or a polypeptide, substantially similar to, or identical with, a region of ICAM-1 capable of binding, associating or otherwise interacting with LFA-1 and thereby inhibiting or reducing ICAM-1-dependent aggregation and/or inhibiting or reducing ICAM-1-dependent effector function of the immune response. The ICAM-1-like peptides or their analogues, therefore, are also capable of binding, associating or otherwise interacting with LFA-1. An amino acid sequence substantially similar to a region of ICAM-1 includes sequences having greater than 70% homology and preferably greater than 80% homology with the selected ICAM-1 region.

The ICAM-1-like peptides may have an amino acid sequence identical with the corresponding sequence in ICAM-1 or may contain amino acid derivatives comprising single or multiple amino acid additions, deletions and/or substitutions compared to the amino acid sequence of the particular region of ICAM-1. The peptides contemplated herein may be chemically synthesized such as by solid phase peptide synthesis or may be prepared by subjecting the ICAM-1 polypeptide to hydrolysis or other chemically

disruptive processes whereby fragments of the molecule are produced. Alternatively, the peptides could be made in vitro or in vivo using recombinant DNA technology. In this case, the peptides may need to be synthesized in combination with other proteins and then subsequently isolated by chemical cleavage or the peptides may be synthesized in multiple repeat units. Furthermore, multiple antigen peptides could also be prepared according to Tam (1988). The selection of a method of producing the subject peptides will depend on factors such as the required type, quantity and purity of the peptides as well as ease of production and convenience.

The use of these peptides in vivo may first require their chemical modification since the peptides themselves may not have a sufficiently long serum and/or tissue half-life. Such chemically modified ICAM-1-like peptides are referred to herein as "analogues". The term "analogues" extends to any functional chemical equivalent of an ICAM-1-like peptide characterized by its increased stability and/or efficacy in vivo or in vitro in respect of the ability to bind, associate or otherwise interact with LFA-1. The term "analogue" is also used herein to extend to any amino acid derivative of the ICAM-1-like peptides as described above.

Analogues of ICAM-1-like peptides contemplated herein include, but are not limited to, modifications to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide synthesis and the use of crosslinkers and other methods which impose conformational constraints on the peptides or their analogues.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an

aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6, trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5'-phosphate followed by reduction with $NaBH_4$.

The guanidino group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation <u>via</u> O-acylisourea formation followed by subsequent derivatisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuric-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids.

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides could be conformationally constrained by, for example, incorporation of C_α and N_α-methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

The ICAM-1-like peptides or their analogues may single length or in tandem or multiple repeats. A single type of peptide or analogue may form the repeats or the repeats may be composed of different molecules including suitable carrier molecules.

The present invention, therefore, extends to peptides or polypeptides and amino acid and/or chemical analogues thereof corresponding to regions of ICAM-1 capable of, or responsible for, binding, associating or otherwise interacting with LFA-1 or parts thereof and thereby inhibit or reduce ICAM-1-dependent aggregation and/or ICAM-1-dependent effector function of the immune response. Reference in the specification and claims herein to "ICAM-1-like peptide" includes all such amino acid and chemical analogues broadly described above.

In one preferred embodiment, the ICAM-1-like peptide corresponds to amino acids in one or more of the regions 330 to 430, 100 to 200 and/or 50-90 of ICAM-1. More preferably, the ICAM-1-like peptides correspond to amino acids in one or more of the regions 367 to 394, 114 to 141 and/or 54 to 82 of ICAM-1. The foregoing amino acid positions are according to Simmons et al (1988).

Even more preferably, the present invention provides the following ICAM-1-like peptides:

JF9, having the amino acid sequence:
VLYGPRLDERDAPGNWTWPENSQQTPMC and its amino acid and chemical analogues;

JF10, having the amino acid sequence:
GGAPRANLTVVLLRGEKELKREPAVGEP and its amino acid and chemical analogues;

and JF14, having the amino acid sequence:
YELSNVQEDSQPMCYSNCPDGQSTAKTFL and its amino acid and chemical analogues.

Peptide JF9 corresponds to positions 367 to 394 of ICAM-1 but Cys-378 is replaced by Ala. The present invention extends to the latter peptide with Cys in position 378, i.e. without the Ala substitution.

The aforementioned peptides inhibit ICAM-1-dependent aggregation and ICAM-1-dependent effector function of the immune response, such as, but not limited to, the inhibition of T-cell mediated killing of the K562 erythroid cell line.

Amino acid analogues of JF9 contemplated herein include, but are not necessarily limited to, the following amino acid sequences shown in Table 1:

Table 1

AMINO ACID SEQUENCE	ICAM-1 SEQUENCE POSITION ACCORDING TO SIMMONS (1988)
VLYGPRLDERD[X]PGNWTWPENSQQTPMC	[367-394]
VLYGPRLDERD[X]	[367-378]
PRLDERD[X]	[371-378]
PGNWT	[379-383]
ERD[X]PGNWT	[375-383]
PRLDERD[X]PGNWT	[371-383]
VLYGPRLDERD[X]PGNWT	[367-383]
GNWTWPENSQ	[380-389]
RD[X]PGNWTWPENSQ	[376-389]
PRLDERD[X]PGNWTWPENSQ	[371-389]
VLYGPRLDERD[X]PGNWTWPENSQ	[367-389]
DERD[X]PG	[374-380]
WPENSQQ	[384-390]
NWTW	[381-384]
NWT	[381-383]
PENSQQTPMC	[385-394]

[X] is A or C

Amino acid analogues of JF10 contemplated herein include but are not limited to the following sequences shown in Table 2:

Table 2

AMINO ACID SEQUENCE	ICAM-1 SEQUENCE POSITION ACCORDING TO SIMMONS (1988)
PRANLTVVLLRGEKELKREPAVGEP	[117-141]
RANLTVVLLRGEKELKREPAVGEP	[118-141]
ANLTVVLLRGEKELKREPAVGEP	[119-141]
NLTVVLLRGEKELKREPAVGEP	[120-141]
LTVVLLRGEKELKREPAVGEP	[121-141]
TVVLLRGEKELKREPAVGEP	[122-141]
VVLLRGEKELKREPAVGEP	[123-141]
RGEKELKREPAVGEP	[127-141]
LKREPAVGEP	[132-141]
GGAPRANLTVVLLRGEKEL	[114-132]
TVVLLRGEKEL	[122-132]
LLRGEKEL	[125-132]
LRGEKEL	[126-132]

Amino acid analogues of JF14 contemplated herein include but are not limited to the following sequences shown in Table 3:

Table 3

ICAM-1 PEPTIDE ANALOGUES OF JF14

CODE	AMINO ACID SEQUENCE	ICAM-1 SEQUENCE POSITION**
JF14:	YELSNVQEDSQPMCYSNCPDGQSTAKTFL	[54-82]
JF14A	YELSNVQEDSQPMCYSNCPDGQSTAK	[54-79]
JF14B	YELSNVQEDSQPMCYSNCPDGQ	[54-75]
JF14C	YELSNVQEDSQPMCYSNC	[54-71]
JF14D	YELSNVQEDSQPMC	[54-67]
JF14E	SNVQEDSQPMCYSNCPDGQSTAKTFL	[57-82]
JF14F	QEDSQPMCYSNCPDGQSTAKTFL	[60-82]
JF14G	QPMCYSNCPDGQSTAKTFL	[64-82]
JF14H	CYSNCPDGQSTAKTFL	[67-82]
JF14I	CYSNCPDGQST	[67-77]
JF14J	NVQEDSQPMC	[58-67]
JF14K	QEDSQPMCYSNCPDGQS	[60-76]
JF14L	QEDSQPMCYSNC	[60-71]
JF14M	QEDSQPMC	[60-67]

**According to sequence described by Simmons et al., 1988.

Although not intending to limit the present invention to any particular mode of action of these peptides, it appears that the peptides span or encompass a functional binding domain of ICAM-1. Peptide JF9 corresponds to a sequence located within the predicted fourth Ig-like domain (D4) on the ICAM-1 molecule (Staunton et al, 1988). Staunton et al, (1989b) have suggested that the LFA-1 binding region of ICAM-1 is located within domains 1 and 2 (D1 and D2) using domain deletion and systematic amino acid substitution techniques. Moreover, the recently defined homologous ICAM-2 molecule has only three Ig-like domains corresponding to D1, D2 and D3 of the ICAM-1 molecule (Staunton et al, 1989b) and lacks the unique region in ICAM-1 defined by the present studies. This incongruity reflects the likelihood that there is more than one binding domain within the ICAM-1 molecule mediating the interaction with LFA-1. However, the inhibitory properties of JF9 show that the region of the ICAM-1 molecule identified herein is an important component of the binding process.

Furthermore, the putative binding domains in JF9 and JF10 to LFA-1 may be different to other LFA-1 binding domains, such as those involved in human immunodeficiency virus infection (Fecondo et al, 1991).

Accordingly, the present invention provides a method for inhibiting ICAM-1-dependent cell adhesion and/or ICAM-1-mediated immune mechanisms in a mammal comprising administering to said mammal an effective amount of an ICAM-1-like peptide or analogue thereof for a time and under conditions sufficient to inhibit or reduce ICAM-1 mediated cell adhesion. Preferably, the mammal is a human or a domestic or ruminant animal. Most preferably, the mammal is a human. The subject method will be particularly useful in the treatment of, inter alia,

graft versus host disease, tissue graft rejection, allergic reaction, inflammatory condition such as labyrinthitis, cancers such as arising from the immune system and possibly infectious agents such as rhinovirus and/or other pathogenic organisms, eg. malaria parasite, where the pathogen interacts with the ICAM-1 target portion of ICAM-1.

The method of administration will vary depending on the circumstances. Examples of such administration would include intravenous injection or infusion. Depending on the particular ICAM-1-like peptide or analogue used, administration by other routes, such as intranasal, oral, intraperitoneal, subcutaneous, rectal or by any means whereby the active molecules can be put in contact with target cells may be possible. Administration may also be topical. The peptides or analogues may have to be modified or co-administered with other molecules to prevent their breakdown or to prolong their half life or to facilitate entry into the bloodstream or target area.

Furthermore, microorganisms such as normal flora (e.g. gut organisms) may be engineered to express and release the ICAM-1 peptides or their analogues of the present invention. The microorganisms may be mutated such that they remain viable but unable to grow. A single length peptide may be secreted or in tandem or multiple repeats. A number of different peptides may also be fused together or fused with a carrier molecule. Examples of suitable organisms include Escherichia coli.

In the above methods of treatment, the ICAM-1-like peptide may also be co-administered or sequentially administered with one or more active agents. Examples of such active agents include but are not limited to cytokines, anti-inflammatory agents and analogues of ICAM-1-like peptides. In particular, the present

invention extends to mixtures of two or more different ICAM-1-like peptides and/or their analogues. Additionally, the ICAM-1-like peptides may be part of a larger molecule where the larger molecule may or may not be related to native ICAM-1. Conveniently, however, the larger molecule is a larger fragment of ICAM-1.

The effective amount of ICAM-1-like peptide or its analogue will be that required to reduce or inhibit ICAM-1-mediated binding, association or other interaction with LFA-1 involved in ICAM-1/LFA-1 cell adhesion and must not be at cytotoxic levels or at least cause only clinically acceptable cytotoxicity. The actual concentration selected will vary depending on the exigencies of the clinical situation but will generally be greater than 0.5 µg/ml and preferably in the range 0.005µM to 200µM. The inclusion of agonists to the ICAM-1-like peptides or their analogues in any therapeutic programme or the inclusion of other molecules capable, for example, of suppressing immune responses, may result in less ICAM-1-like peptide or analogue being required. In such a case, 5nM to 100µM of ICAM-1-like peptide or analogue may be sufficient.

Another aspect of the present invention is directed to pharmaceutical compositions comprising an ICAM-1-like peptide and/or its analogue and one or more pharmaceutically acceptable carriers and/or diluents. A convenient reference for the preparation of pharmaceutical compositions including suitable carrier vehicles and their formulation, inclusive of other human proteins, eg. human serum albumin, is described in Remington's Pharmaceutical Sciences 17th ed., Mack Publishing Co., edited by Osol et al., which is hereby incorporated by reference.

The pharmaceutical compositions may also include additional molecules to stabilize the active agent or which act as agonists or otherwise assist the ICAM-1-peptide and/or its analogue to perform the desired function.

In one embodiment, the ICAM-1-peptide and/or its analogue will be in combination with another active agent which may inhibit cell adhesion and/or immune response mechanisms.

Furthermore, the ICAM-1-like peptide or analogue thereof may be used in the manufacture of a medicament for the treatment of diseases arising from ICAM-1/LFA-1 cell adhesion such as, but not limited to, graft versus host disease, tissue graft rejection, cancer, severe allergic reaction and/or infection by pathogenic agents whose infectivity depends upon the function or integrity of ICAM-1, examples are the malaria parasites and rhinoviruses.

The present invention also extends to the use of ICAM-1-like peptides and/or analogues thereof to quantitatively or qualitatively detect or screen for LFA-1 receptors on cells in the blood stream or other body fluids. A range of techniques is available which could use the above molecules directly or indirectly via antibodies to same, such as ELISA or radioimmuno assay. The ICAM-1-like peptide and/or its analogue, may also be used in immobilized form to isolate specific LFA-1 expressing cells. The present invention also extends to monoclonal or polyclonal antibodies to the ICAM-1-like peptides or their analogues.

Further in accordance with the present invention, there is, provided a method for determining functional binding domains in polypeptides or proteins. The ICAM-1-like peptide JF9 was identified using this method. It is not the intention, however, to limit JF9 in any way to this method, nor should the applicability of this method be in any way limited to JF9 or ICAM-1-like peptides.

Accordingly, this aspect of the present invention is directed to a method of fingerprinting one or more functional domains in a polypeptide or protein, comprising identifying one or more sequences of amino acids in the hydrophilic portion of said polypeptide or protein which are substantially absent in a functionally distinct but structurally similar, second polypeptide or protein.

The term "fingerprinting" is used in its widest sense and includes characterising, identifying or otherwise determining a particular region of a polypeptide or protein. The term is particularly used herein to refer to a target sequence of amino acids. By "functional domain" is meant the one or more continuous sequences of amino acids in a polypeptide or protein which, either separately or in combination with the remainder of the polypeptide or protein, including amino acid sequence and/or tertiary structure, are responsible for or contribute to a particular activity of function associated with the polypeptide or protein or parts thereof. For example, the functional domain of a protein ligand may be its receptor binding site. In a particular non-limiting example described herein, the functional domains of interest in respect of the ICAM-1 molecule are those involved in adhesion of ICAM-1 to LFA-1. By a "functionally distinct" polypeptide or protein means one with a similar amino acid sequence but with a different function relative to another polypeptide or protein.

The hydrophilic portions of the polypeptide or protein may be determined by a number of known techniques. One convenient method is the Kyte-Doolittle algorithm for predicting potential hydrophilic binding regions (Kyte and Doolittle, 1982). In relation to membrane or cell wall associated or embedded polypeptides or proteins, these polar regions are likely to be surface exposed. Hydrophilic regions have negative hydropathy indices.

In accordance with the present invention, a functional domain is putatively identified in the hydrophilic region when said hydrophilic region contains at least one continuous sequence of amino acids substantially absent in a functionally distinct second polypeptide or protein under consideration. Such a comparison of amino acid sequences may be accomplished by any number of procedures such as, but not limited to a dot-matrix sequence comparison which shows simple identity versus non-identity scoring of amino acids. A functional domain is, therefore, putatively identified by finding an amino acid sequence in the polypeptide or protein of interest with essentially no identity in the functionally distinct polypeptide or protein.

Furthermore, the order by which the steps of the subject of method is performed may be altered such that the continuous amino acid sequences in a polypeptide or protein of interest may first be identified and then a hydrophobicity plot conducted to locate which of these sequences correspond to a predicted hydrophilic (e.g. polar surface) region.

Accordingly, another aspect of the present invention relates to a method for fingerprinting a functional domain in a polypeptide or protein which method comprises first identifying one or more continuous non-identical amino acid sequences in said first polypeptide or protein

absent in a second functionally distinct but structurally similar polypeptide or protein, identifying likely surface exposed regions of said first polypeptide or protein and then determining whether said one or more amino acid sequences occurs in said surface exposed regions.

Apart from the functional significance of these observations with respect to ICAM-1 and in particular JF9, the approach used in accordance with the present invention has general application for the identification of functional domains in structurally similar proteins which do not share the same biological activity. The subject method relies on two assumptions: (a) the functional domain(s) are likely to be hydrophilic in nature and (b) there is no significant sequence identity in the domain to other homologous but functionally distinct proteins.

The present invention is further described by the following non-limiting Figures and Example.

In the Figures:

Figure 1 is a graphical representation showing a structural and comparative analysis of ICAM-1. (a) A hydropathy plot of ICAM-1 using the method of Kyte and Doolittle (1982) with a window setting of 7 residues; (b) A dot matrix sequence comparison of ICAM-1 (Staunton et al., 1988, Simmons et al., 1988.) using the method of Maizel and Lenk (1981). The sequences were compared for simple identity versus non-identity scoring of amino acids with a comparison length setting of 10 amino acids and a minimum match score of 4. The lines extending from the dot matrix plot to the hydropathy plot indicate the correspondence between the region of non-identity within the ICAM-1 sequence to a predicted polar and thus likely

surface-exposed region of the ICAM-1 molecule.

Figure 2 is a photographic representation depicting inhibition studies of ICAM-1/LFA-1 dependent homotypic aggregation of Raji cells. (a) Raji cells with no peptide present; (b) inhibition of Raji cell aggregation with 20 µg/ml of purified WEHI-CAM-1 antibody; (c) inhibition of Raji cell aggregation by 80 µg/ml JF9 peptide; and (d) Raji cells in the presence of a control peptide, 500 µg/ml JF7B. All assays were performed by mixing HPLC-pure, sterile peptides (0 - 500 µg/ml) with 50,000 Raji cells in RPMI 1640/5% (v/v) foetal calf serum in microtitre plate wells and assessing aggregation by microscopy over a 24 hour period. All scoring was performed by an observer who was blinded to the experimental design.

Figure 3 is a graphical representation showing inhibition of cytotoxic T cell activity by the JF9 peptide. T cell-mediated killing of K562 cells was measured by the release of ^{51}Cr into the medium as previously described. (Boyd et al, 1988) (a) negative control, no peptide or antibody present; (b) positive control, 20 µg/ml of purified WEHI-CAM-1 antibody added; (c) negative control antibody, 20 µg/ml of purified WEHI-B2 antibody added; (d) 100 µg/ml of JF7B added; (e) 100 µg/ml of JF9 added; (f) 100 µg/ml of JF13A added. T cells were activated in a two way mixed lymphocyte response between two normal, unrelated donors. The cells (10^6/ml) were mixed in a 1:1 ratio and cultured in RPMI 1640/10% (v/v) FCS for 4 days. Recombinant interleukin 2 (Cetus Corporation) was added (200 U/ml) and the cultures continued for a further 3 days. Cells were harvested and added to a constant number (10^4 cells/well) of ^{51}Cr-labelled K562 cells in 96 well microtitre tray. ^{51}Cr release was measured after 4 hours.

EXAMPLE

The following example relates to the identification of functional binding regions in ICAM-1, the synthesis of peptides based thereon and their use to inhibit ICAM-1-mediated adhesion to cells which express LFA-1.

Peptides were synthesised using a highly optimised protocol (Clark-Lewis and Kent, 1989), coupling t-Boc amino acids to 4-methylbenzhydrylamine resin or 4-(oxymethyl) phenylacetamidomethyl-t-Boc-L-Pro resin with an average coupling efficiency of greater than 99.3% per cycle as determined by quantitative ninhydrin analysis (Sarin et al, 1981). Following standard HF cleavage from the resins, all peptides were purified by reverse phase HPLC, using an Aquapore C8 100 x 10 mm 20 micron Prep 10 cartridge column and Aquapore RP-300 30 x 4.6 mm 7 micron cartridge columns (Applied Biosystems, Santa Clara, CA).

The ICAM-1 sequence was analysed using the Kyte-Doolittle (1982) algorithm for predicting potential hydrophilic binding regions. In Figure 1(a), it can be seen that there are a number of predicted hydrophilic regions with hydropathy indices as low as -3.4, and clustering of both positive and negative charges. These polar regions are likely to be surface exposed.

The next step was to define which of these regions may be functional binding sites. To do this, unique regions were predicted by a dot-matrix sequence comparison of ICAM-1 with its functionally distinct homologue, MAG. Simple identity versus non-identity scoring of amino acids is shown in the dot matrix plot in Figure 1(b). A detailed analysis of the resulting plot revealed a region within the ICAM-1 sequence which had essentially no identity with MAG. This region spanned amino acid

residues 369 to 391 and is indicated by a virtual absence of dots or stings of dots on the plot shown in Figure 1(b). When this sequence was analysed in conjunction with the hydrophobicity plot data, there was a striking correspondence to a predicted polar surface region of the ICAM-1 sequence.

The uniqueness of this region of ICAM-1 (amino acid residues 369-391) was further confirmed by FASTA short sequence homology searches (Pearson et al, 1988) of a number of databases, including the NBRF (Version 23), NBRF Provisional (Version 41), SWISSPROT (Version 13), GenBank [Translated] (Version 62) and PRF - Japan (version 90/01) with stringency set initially at ktup = 2, followed by ktup = 1. These searches revealed no protein sequences with any significant identity to the ICAM-1 subsequence. The best alignment was 50% identity in a 14 amino acid overlap with the HIV-1 env polyprotein precursor. No other significant matches were found.

It was predicted that this surface-exposed ICAM-1 subsequence may have a role in the adhesion properties of this protein. To experimentally test this prediction, a peptide analogue spanning this sequence (JF9: amino acid positions 367-394 with Cys-378 replaced with Ala; Table I) and two control peptides of similar size, corresponding to the N and C-termini (JF7B: amino acid positions 1 - 23 and JF13A: amino acid positions 479 - 507 respectively; Table 4) were prepared by total chemical synthesis. These peptides were tested for their ability to interfere with ICAM-1-mediated adhesion in several functionally distinct biological systems.

Previous studies have shown that the WEHI-CAM-1 anti-ICAM-1 monoclonal antibody completely inhibited the homotypic aggregation of the Raji Burkitt lymphoma cell line (Boyd et al, 1988). This effect can be reproduced

with anti-LFA-1 antibodies (Marlin and Springer, 1987) implying that the ICAM-1/LFA-1 interaction is the most important adhesion interaction for Raji cell aggregation. Testing of all three synthetic peptides (Figure 2) over a concentration range of 0 - 500 µg/ml in the aggregation assay (Figure 2) indicated that the ICAM-1-like peptide JF9, based on the "unique" sequence, completely inhibited Raji cell aggregation at a concentration as low as 24 µM while the two control peptides had no detectable inhibitory effect, even at concentrations of 500 µg/ml (220µM). A time course study of the inhibition of cell aggregation by JF9 suggested that the peptide still had significant inhibitory activity after 48 hours. The same pattern of inhibition was observed when the set of peptides were tested in the same assay with TPA-induced U937 cells, a T cell line which shows inducible expression of ICAM-1 following treatment with TPA (Rothlein et al, 1988; Boyd et al, 1989a). These results indicate that the JF9 peptide specifically inhibits ICAM-1 dependent aggregation but not ICAM-1 independent aggregation of cells.

Furthermore, testing of the JF9 peptide and control peptides in a cytotoxic T cell assay over a concentration range of 0 to 250 µg/ml demonstrated that JF9 was able to significantly inhibit T-cell mediated killing of the K562 erythroid cell line effectively at a concentration as low as 30uM (100µg/ml) (Figure 3).

TABLE 4

ICAM-1-LIKE PEPTIDE AMINO ACID SEQUENCES	
JF7B:	NAQTSVSPSKVILPRGGSVLVTC
JF9:	VLYGPRLDERDAPGNWTWPENSQQTPMC
JF10:	GGAPRANLTVVLLRPGKELKREPAVGEP
JF13A:	NRQRKIKKYRLQQAQKGTPMKPNTQATPP

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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CLAIMS:

1. An ICAM-1-like peptide or an analogue thereof capable of inhibiting or reducing adhesion between cells in a mammal.

2. The ICAM-1-like peptide or a analogue thereof according to claim 1 wherein said peptide or analogue corresponds in whole or in part to one or more regions of ICAM-1 capable of, or responsible for, binding, associating or otherwise interacting with LFA-1 or parts thereof wherein said ICAM-1-like peptide or its analogue is capable of inhibiting or reducing ICAM-1-dependent aggregation and/or ICAM-1-dependent effector function of the immune response.

3. The ICAM-1-like peptide or analogue thereof according to claim 2 wherein said peptide or analogue corresponds in whole or in part to the amino acid sequence in one or both of the amino acid regions 330 to 430, 100 to 200 and/or 50 to 90 of ICAM-1.

4. The ICAM-1-like peptide or analogue thereof according to claim 3 wherein said peptide or analogue corresponds in whole or in part to the amino acid sequence in one or both of the amino acid regions 367 to 394, 114 to 141 and/or 54 to 82 of ICAM-1.

5. The ICAM-1-like peptide or analogue thereof according to claim 4 wherein said peptide or analogue corresponds in whole or in part to the amino acid sequence:

VLYGPRLDERD[X]PGNWTWPENSQQTPMC,

wherein [X] is A or C.

6. The ICAM-1-like peptide or analogue thereof according to claim 5 wherein [X] is A.

7. The ICAM-1-like peptide or analogue thereof according to claim 4 wherein said peptide or analogue corresponds in whole or in part to the amino acid sequence:

GGAPRANLTVVLLRGEKELKREPAVGEP.

8. The ICAM-1-like peptide or analogue thereof according to claim 4 wherein said peptide or analogue corresponds in whole or in part to the amino acid sequence:

YELSNVQDSQPMCYSNCPDGQSTAKTFL.

9. The ICAM-1-like peptide or analogue thereof according to claim 4 corresponding to any one or more of the amino acid sequences shown in Table 1.

10. The ICAM-1-like peptide or analogue thereof according to claim 7 corresponding to any one or more of the amino acid sequences shown in Table 2.

11. The ICAM-1-like peptide or analogue thereof according to claim 8 corresponding to any one or more of the amino acid sequences shown in Table 3.

12. The ICAM-1-like peptide or analogue thereof according to anyone of the preceding claims wherein the mammal is a human, ruminant animal or domestic animal.

13. The ICAM-1-like peptide or analogue thereof according to claim 12 wherein in mammal is a human.

14. A method for inhibiting ICAM-1-dependent cell adhesion and/or ICAM-1-mediated immune mechanisms in a mammal comprising administering to said mammal an effective amount of an ICAM-1-like peptide or an analogue thereof for a time and under conditions sufficient to inhibit or reduce ICAM-1 mediated cell adhesion.

15. The method according to claim 14 wherein said ICAM-1-like peptide or analogue thereof corresponds in whole or in part to one or more regions of ICAM-1 capable of, or responsible for, binding, associating or otherwise interacting with LFA-1 or parts thereof wherein said ICAM-1-like peptide or its analogue is capable of inhibiting or reducing ICAM-1-dependent aggregation and/or ICAM-1-dependent effector function of the immune response.

16. The method according to claim 15 wherein said peptide or analogue corresponds in whole or in part to the amino acid sequence in one or both of the amino acid regions 330 to 430, 100 to 200 and/or 50 to 90 of ICAM-1.

17. The method according to claim 16 wherein said peptide or analogue corresponds in whole or in part to the amino acid sequence in one or both of the amino acid regions 367 to 394, 114 to 141 and/or 54 to 82 of ICAM-1.

18. The method according to claim 17 wherein said peptide or analogue corresponds in whole or in part to the amino acid sequence:

VLYGPRLDERD[X]PGNWTWPENSQQTPMC,

wherein [X] is A or C.

19. The method according to claim 18 wherein [X] is A.

20. The method according to claim 17 wherein said peptide or analogue corresponds in whole or in part to the amino acid sequence:

GGAPRANLTVVLLRGEKELKREPAVGEP.

21. The method according to claim 17 wherein said peptide or analogue corresponds in whole or in part to the amino acid sequence:

YELSNVQDSQPMCYSNCPDGQSTAKTFL.

22. The method according to claim 18 wherein the peptide or analogue thereof corresponds to any one or more of the amino acid sequences shown in Table 1.

23. The method according to claim 20 wherein the peptide or analogue thereof corresponds to any one or more of the amino acid sequences shown in Table 2.

24. The method according to claim 21 wherein the peptide or analogue thereof corresponds to any one or more of the amino acid sequences shown in Table 3.

25. The method according to anyone of claims 14 to 23 wherein the mammal is a human, domestic animal or ruminant animal.

26. The method according to claim 25 wherein the mammal is a human.

27. The method according to claim 14 wherein administration is by the intravenous, infusion, oral, intranasal, intraperitoneal, subcutaneous, rectal or topical route.

28. The method according to claim 14 wherein the administration is by the secretion of said ICAM-1-like peptide or analogue thereof from a microorganism present in the mammal.

29. The method according to claim 28 wherein the microorganism is Escherichia coli.

30. The method according to claim 27 wherein the administration is by the intravenous or infusion route.

31. The method according to claim 14 further comprising the co-administration or sequential administration of one or more other active agents.

32. The use of the ICAM-1-like peptides or analogues thereof hereinbefore described in the manufacture of a medicament for the treatment of disease arising from ICAM-1/LFA-1 cell adhesion.

33. The use according to claim 32 wherein the disease includes graft versus host disease, tissue graft rejection, cancer, allergic reaction, inflammatory condition and/or infection by pathogenic agents whose infectivity depends on the functions or integrity of ICAM-1.

34. The use according to claim 33 wherein the inflammatory condition is labyrinthitis.

35. A pharmaceutical composition comprising an ICAM-1-like peptide or an analogue thereof according to anyone of claims 1 to 13 and one or more pharmaceutical carriers and/or diluents.

36. The composition according to claim 35 further comprising one or more other active components.

37. The composition according to claim 36 wherein the other active components comprise anti-inflammatory agents, cytokines and/or analogues of ICAM-1-like peptides.

38. A method for fingerprinting one or more functional binding domains in a polypeptide or protein comprising identifying one or more sequences of amino acids in the hydrophilic portion of said polypeptide or protein which are substantially absent in a functionally distinct but structurally similar second polypeptide or protein.

39. The method according to claim 38 wherein said polypeptide or protein contains a receptor binding site and said functional domain is said site.

40. The method according to claim 38 or 39 wherein the polypeptide or protein is ICAM-1 and the functional domain is that region involved in adhesion of ICAM-1 to LFA-1.

41. A method for fingerprinting a functional domain in a polypeptide or protein which method comprises first identifying one or more continuous non-identical amino acid sequences in said first polypeptide or protein absent in a second functionally distinct but structurally similar polypeptide or protein, identifying likely surface exposed regions of said first polypeptide or protein and then determining whether said one or more amino acid sequences occurs in said surface exposed regions.

42. The method according to claim 41 wherein said polypeptide or protein contains a receptor binding site and said functional domain is said site.

43. The method according to claim 41 or 42 wherein said polypeptide or protein is ICAM-1 and said functional domain is that regions involved in adhesion of ICAM-1 to LFA-1.

44. An antibody to the ICAM-1-like peptide or analogue thereof according to any one of claims 1 to 13.

45. The antibody according to claim 44 wherein said antibody is a monoclonal antibody.

46. The antibody according to claim 44 wherein said antibody is a polyclonal antibody.

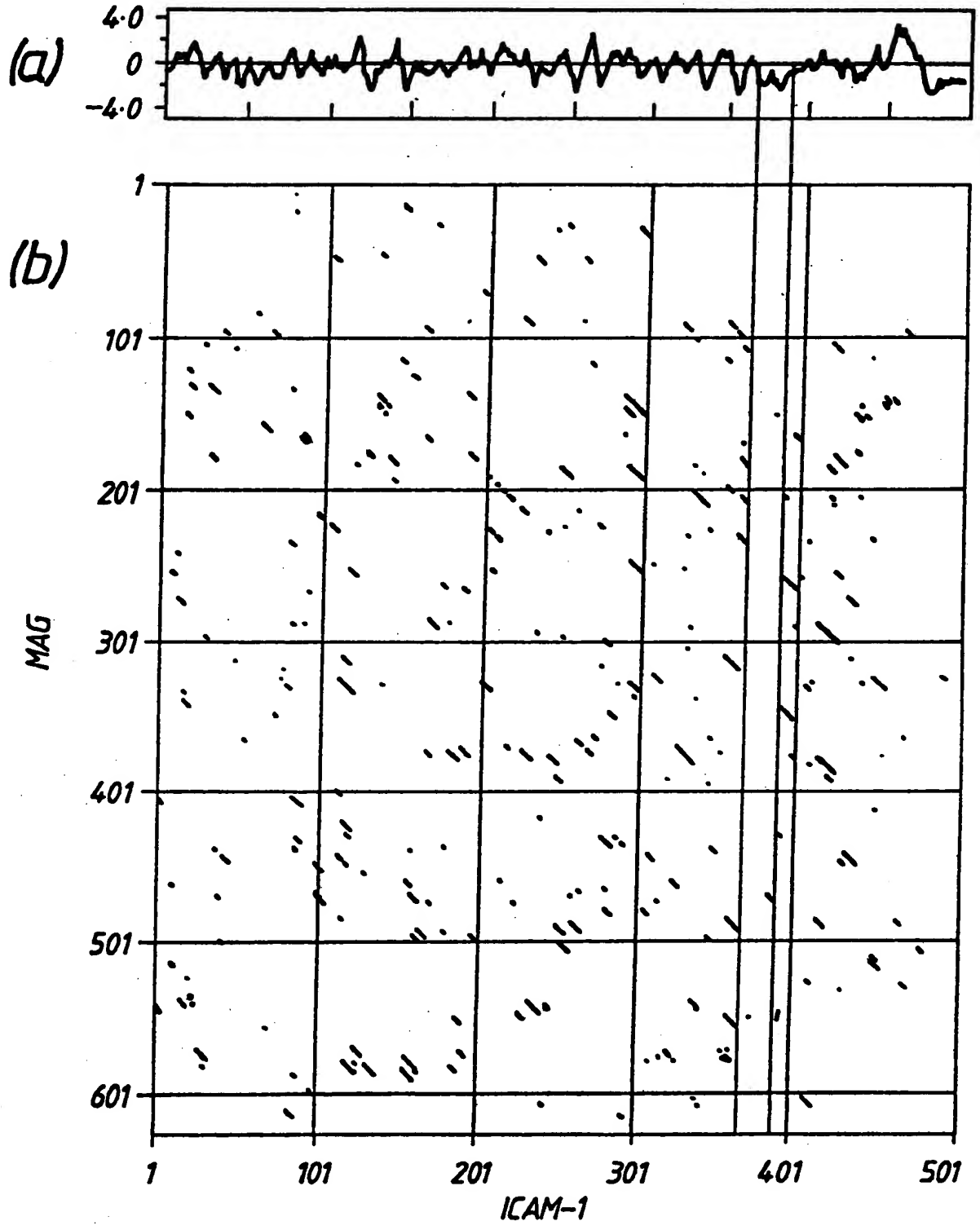
(a)
4·0
0
−4·0
(b)
1
101
201
301
401
501
601
MAG
1
101
201
301
401
501
ICAM-1

Fig.1.

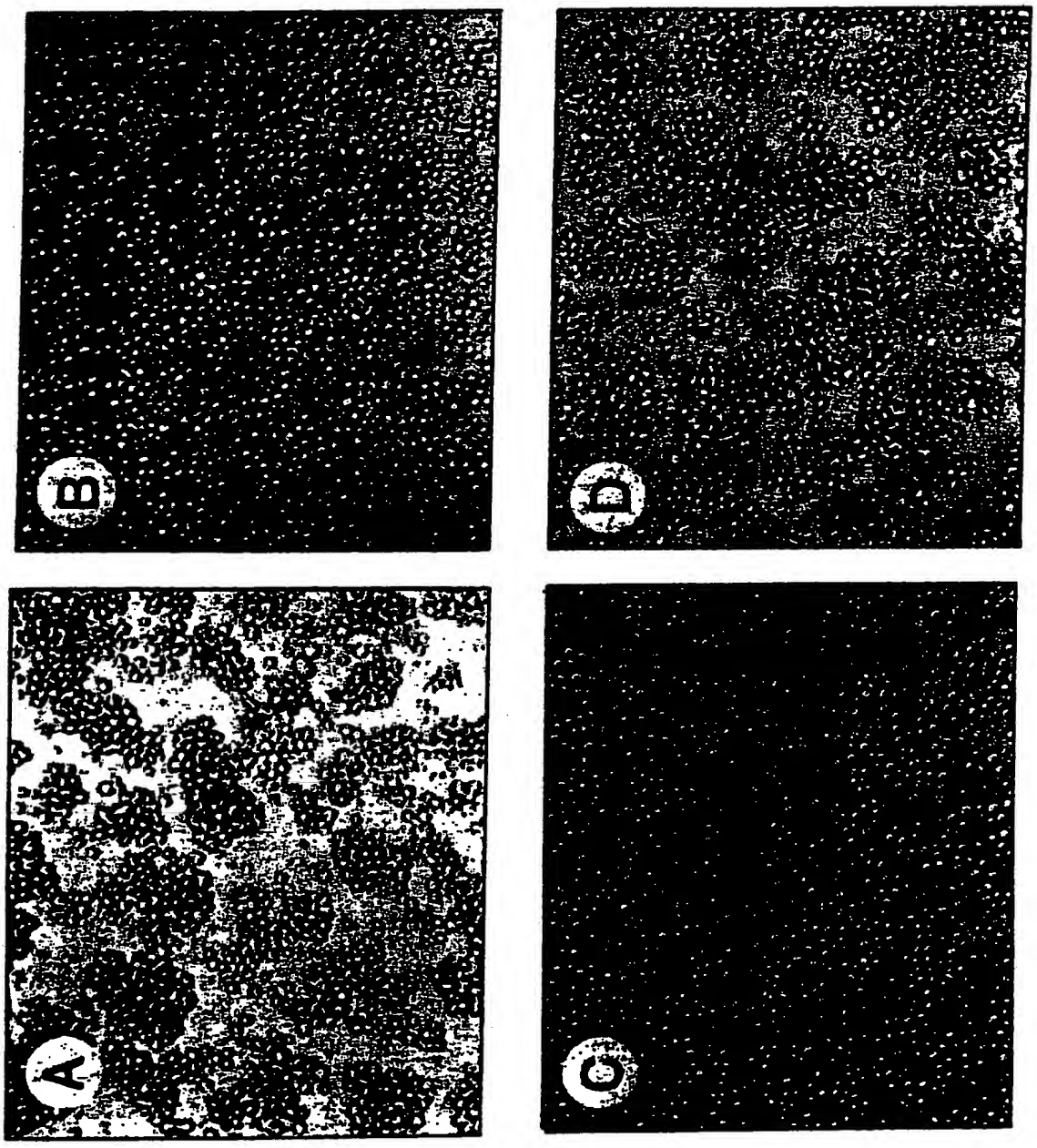

Fig. 2.

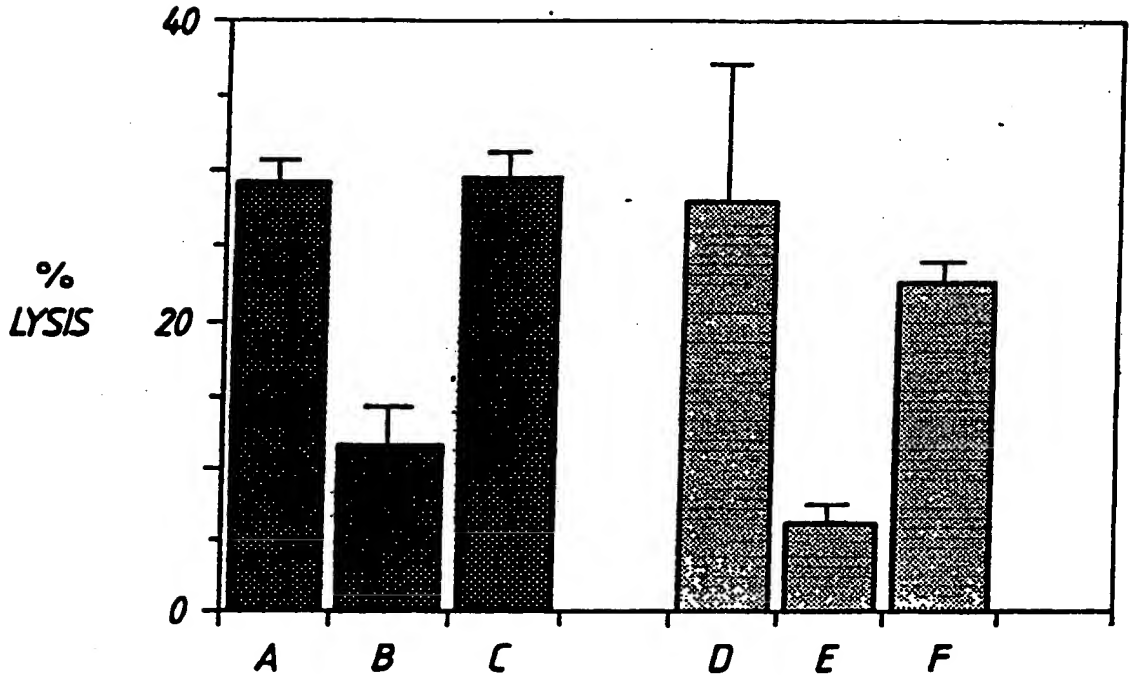

Fig.3.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 91/00205

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[5] C07K 5/08, 5/10, 7/06, 7/08, 7/10, 13/00, 15/12, 15/14, C12P 21/08, A61K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
	DERWENT WPI/WPIL; CHEMICAL ABSTRACTS Keywords: "INTERCELLULAR()ADHESION" OR "ICAM:"

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AU: C07K 5/08, 5/10, 7/06, 7/08, 7/10, 13/00, 15/14, C07C 103/52
CHEMICAL ABSTRACTS: STN CAS-ONLINE PROTEINSEQUENCE SEARCH

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X 204	Journal of Immunology, volume 137(4), pages 1270-1274. 1986 (ROTHLEIN) "A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1"	(1, 2, 12-15, 25-37, 44-46)
X,Y 204	European Journal of Immunology, volume 18, pages 35-39. 1988 (DOUGHERTY et al) "The function of human intercellular adhesion molecule-1 (ICAM-1) in the generation of an immune response"	(1, 2, 12-15, 25-37, 44-46)
X 204	Cell, volume 52, pages 925-933, 1988 (STAUNTON et al) "Primary Structure of ICAM-1 demonstrates interaction between members of the Immunoglobulin and Integrin Supergene families."	(1-4, 7, 10)
X	Chemical Abstracts Registry No 134448-230 STN CAS-ONLINE REGISTRY FILE PROTEIN SEQUENCE	(3, 4, 5, 6, 9)
X	Journal of Molecular Biology, volume 157, (1982) pages 105-132 (KYTE & DOOLITTLE) "A simple Method for Displaying the Hydropathic Character of a Protein"	(40, 43)
X	Science, volume 247, 26 January 1990 pages 456-459 (WEGNER et al)	(1-2, 12-15, 25-37, 44-46)

Ref. PCT/AU91/00204

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
3 September 1991 (03.09.91)	9 September 91
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	A W BESTON

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
P,X	European Journal of Immunology, volume 20, pages 2591-2596 (GEISSLER) "Amonoclonal antibody directed against the human intercellular adhesion molecule (ICAM-1) modulates the release of tumor necrosisfactor-oc, interferon g, and interleukin".	(1-2, 12-15, 25-37, 44-46)
X 204	AU,A, 15509/88 (BAYLOR COLLEGE OF MEDICINE & BOEHRINGER INGELHEIM PHARMACEUTICALS, INC) 27 July 1989 (27.07.89)	(1-2, 12-15, 25-37, 44-46)
X 204	AU,A, 15518/88 (DANA FARBER CANCER INSTITUTE), 10 November 1988 (10.11.88) entire document, especially note tables 5, 6, 8, 9	(1-4, 7, 8, 10-17, 20-21, 23-37, 40, 43-46)
X 204	AU,A, 26332/88 (DANA FARBER CANCER INSTITUTE), 18 May 1989 (18.05.89)	(1-4, 7, 8, 10-17, 20-21, 23-37, 44-46)
X 204	AU,A, 26333/88 (BAYLOR COLLEGE OF MEDICINE & BOEHRINGER INGELHEIM PHARMACEUTICALS, INC) 27 July 1989 (27.07.89)	(1-2, 12-15, 25-37, 44-46)
X 204	AU,A, 29473/89 (THE GENERAL HOSPITAL CORPORATION) 22 September 1989 (22.09.89) especially pages 19, 91-96	(1-4, 12-17, 27-33, 35-37, 39, 43-46
X 204	AU,A, 44128/89 (DANA FARBER CANCER INSTITUTE) 18 April 1990 (18.04.90)	(1-4, 78, 10-17, 20-21, 23-37, 40, 43-46)
P,X 204	AU,A, 48767/90 (MOLECULAR THERAPEUTICS, INC) 2 August 1990 (02.08.90) especially pages 5, 6, 10 and claims	(1, 2, 12-15, 25-27, 31-37, 44-46)

See also PCT/AU91/00204

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [ ] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.[ ] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2.[ ] Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.[ ] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [X] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

Claims 1-37, 40, 43-46

Claims 38, 39, 41, 42

1.[ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.[ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.[x] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-37, 40, 43-46

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.